J. Clin. Chem. Clin. Biochem.
Vol. 25, 1987, pp. 511–514

A New, Very Sensitive, Bioluminescence-Enhanced Detection System for Protein Blotting

Ultrasensitive Detection Systems for Protein Blotting and DNA Hybridization, I.

By *Regina Hauber* and *R. Geiger*

Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München

(Received April 15, 1987)

Summary: A relatively simple, very sensitive bioluminescence-enhanced detection system for protein blots is described. The method utilizes antibodies conjugated with alkaline phosphatase. The alkaline phosphatase then takes part in a reaction by releasing *D*-luciferin (*Photinus pyralis*) from *D*-luciferin-O-phosphate. Liberated *D*-luciferin reacts with luciferase, ATP and oxygen with light emission. The light is detected by a sensitive photographic film, thereby permitting the visualization of the alkaline phosphatase-conjugated antibodies. Under non-optimized conditions the limit of detection is at present 5 to 50 pg of protein, corresponding e. g. to 30 to 300 $\times 10^{-18}$ mol of rabbit immunoglobulin G. The detection system is therefore 100 times more sensitive than other systems used at present.

Introduction

Protein blot analyses are now extensively used for research and for diagnosis of infectious diseases, e. g. hepatitis or acquired immunodeficiency syndrome (AIDS) (1–6). The first practical methods for transfer of proteins from gel electropherograms to cellulose or nitrocellulose were published in 1979 (7–9) and since that time there has been an explosive increase in applications of these techniques (for a review see l. c. (10)). In the beginning, radioactive labels were used to increase the sensitivity of antigen detection (7, 8, 11, 12). During recent years non-radioactive labels such as horseradish peroxidase[1]) or alkaline phosphatase[1]) have been introduced (13–21), which allow a comparable sensitivity of detection. As little as 1 to 10 ng of protein can be detected under optimal conditions.

In this communication we describe the development of a bioluminescence-enhanced detection system with a greatly increased sensitivity of detection for protein blotting.

Materials and Methods

Synthetic *D*-luciferin (*Photinus pyralis*), ATP, alkaline phosphatase (calf intestine; 200–400 U/mg; 25 °C) and rabbit immunoglobulin G were purchased from Sigma, Taufkirchen, FRG.

Human urinary kallikrein[1]) and porcine leukocyte elastase[1]) were isolated as described by *Geiger* et al. (22, 23). Luciferase[1]) (*Photinus pyralis*; spec. act. 8 mU/mg) was a product of Boehringer, Mannheim, FRG.

D-luciferin-O-phosphate was synthesized as described in l. c. (24). The substrate is commercially available from Novabiochem AG, CH-4448 Läufelfingen, Switzerland.

Antibodies against human urinary kallikrein, porcine leukocyte elastase and rabbit immunoglobulin G were raised in rabbits and goats as described in l. c. (25). Anti-rabbit IgG goat immunoglobulin alkaline phosphatase conjugate was synthesized as described by *Geiger* et al. (26).

Nitrocellulose filters (BA 85, 0.45 μm) were products of Schleicher and Schüll, Dassel.

[1]) Enzymes:
Alkaline phosphatase (EC 3.1.3.1);
human tissue (urinary) kallikrein (EC 3.4.21.35);
porcine polymorphonuclear leukocyte elastase (EC 3.4.21.37, formerly EC 3.4.21.11);
horse radish peroxidase (EC 1.11.1.7);
luciferase (EC 1.13.12.7).

Photographic film, Tri X pan, 380 ASA, was purchased from Kodak AG, Stuttgart, and developed using a procedure given by the manufacturers.

Protein transfer or protein blotting was performed as described in l. c. (27) and (28).

Bioluminescent detection of immunoblotted proteins

Protein was transferred to nitrocellulose soaked with phosphate buffer (6.5 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 137 mmol/l NaCl, 2.7 mmol/l KCl, pH 8.0) as described in l. c. (27) and (28). Nitrocellulose was then blocked with 50 g/l bovine serum albumin in phosphate buffer for 2 h at 25 °C. Then the filter was incubated for 2 h at 25 °C in phosphate buffer containing anti-rabbit IgG alkaline phosphatase conjugate (1 : 500 dilution of conjugate in phosphate buffer). Thereafter the filter was washed 3 times with phosphate buffer without conjugate and transfered to a transparent plastic dish containing about 6 ml of the detection solution (40 mmol/l HEPES buffer, 1.6 mmol/l diethanolamine, 6 mmol/l $MgCl_2$, 0.54 mmol/l EDTA, 3.4 mmol/l dithiothreitol, 2.6 mmol/l ATP, 2 mmol/l luciferin-O-phosphate, 0.15 mg luciferase, pH 8.0). The dish was placed on a photographic film in the dark for 2 h. After development (5 min at 25 °C in a solution of Kodak HC-110) and fixation of the film, the position of protein binding antibody was revealed as dark spots.

Results and Discussion

We report here an alternative, sensitive, bioluminescence-enhanced detection method of protein blotting. The principle of bioluminescent protein blotting is shown in figure 1. After application of proteins to a nitrocellulose filter, either by direct application or by electric transfer, an antibody alkaline phosphatase conjugate is added. Protein-bound alkaline phosphatase conjugate liberates luciferin from the substrate luciferin-O-phosphate, and luciferin is oxidized by luciferase (*Photinus pyralis*) under light emission (fig. 2). Emitted light exposes a sensitive photographic film. Thus specifically bound alkaline phosphatase conjugate can be visualized after development.

In the detection step, alkaline phosphatase and luciferase (*Photinus pyralis*) must both be active in the same buffer solution. The pH optima of these enzymes, pH 7.75 for luciferase (*Photinus pyralis*) and pH 9.6 for alkaline phosphatase are different. Based on the results depicted in figure 3, a buffer of pH 8.0 was chosen, where alkaline phosphatase and luciferase are still sufficiently active.

Since commercial detection equipment is not available, we used a home-made device, as shown in figure 4.

Figure 5 shows an example of a photographic film, where even spots corresponding to 5 pg protein (rabbit immunoglobulin G) can be detected.

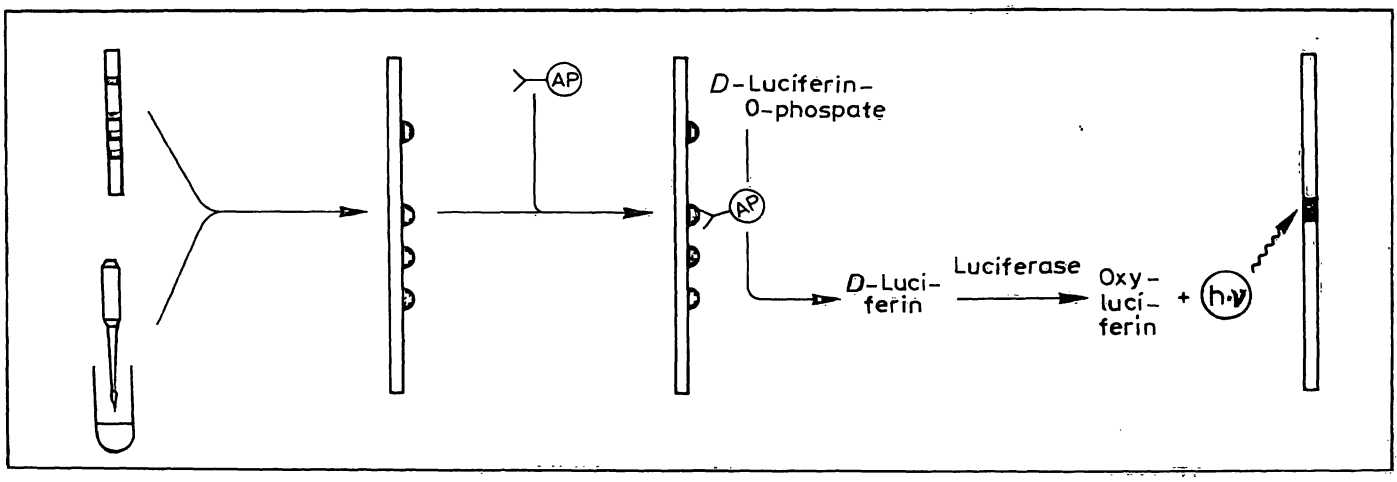

Fig. 1. Scheme of the bioluminescence-enhanced detection system of protein blotting. ⟩—(AP) = antibody alkaline phosphatase conjugate.

1) *D*-Luciferin-O-phosphate —(Alk. Phosphatase)→ *D*-Luciferin + P_i

2) *D*-Luciferin + O_2 + ATP —(Luciferase, (Mg^{2+}))→ Oxyluciferin + h · ν + AMP + PP_i

Fig. 2. Scheme of bioluminescence reaction using firefly luciferase (*Photinus pyralis*).

The detection system has been successfully used for the following antigens: rabbit immunoglobulin G, human urinary kallikrein and porcine leukocyte elastase. The detection system is at present not optimized with respect to sensitivity of photographic film and exposure time, but enzyme activities are stable for two hours using the detection buffer described above. Limits of detection determined for the present method are between 5 and 5000 pg (corresponding to 30 $\times$ 10^{-18} mol of rabbit immunoglobulin G, to 10^{-16} mol of human urinary kallikrein and to 7 $\times$ 10^{-16} mol of porcine leukocyte elastase) at an exposure time of 2 hours. Taking into account that the measurement of light is performed under non-optimal conditions (only exposure of photographic film, no use of electronical photomultipliers or scanners) the detection limits can be lowered by powers of ten even at a shorter exposure time of the film. By the use of sensitive electronical detection systems, e. g. a photomultiplier or a luminometric scanner, protein samples could be measured by the same principle in the femtogram range (< 10^{-18} mol), as demonstrated recently for bioluminescence-enhanced immunoassays (24, 26).

Very sensitive detection systems are nowdays of special interest. By their use many problems in science and diagnosis could be solved more rapidly and at an earlier stage, such as the quick determination of low concentrations of proteins or peptides produced by recombinant techniques in microorganisms or culture broth (29) or the determination of low antibody titers in the first stages of antibody formation, e. g. in diseases like acquired immunodeficiency syndrome (AIDS).

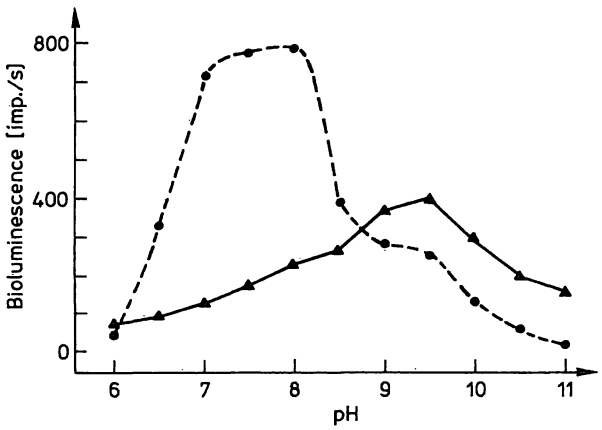

Fig. 3. pH optima of calf intestine alkaline phosphatase (▲) and firefly (*Photinus pyralis*) luciferase (●). For details, see l. c. (24).

Acknowledgement

We wish to thank Prof. Dr. *H. Fritz* for his support during this work. We thank Dr. *F. Fiedler* for reading over the manuscript.

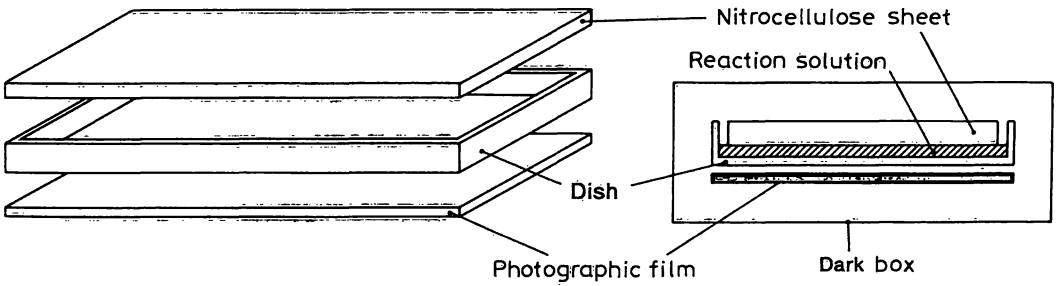

Fig. 4. Scheme of the device used for the visualization of the bioluminescence-enhanced detection system.

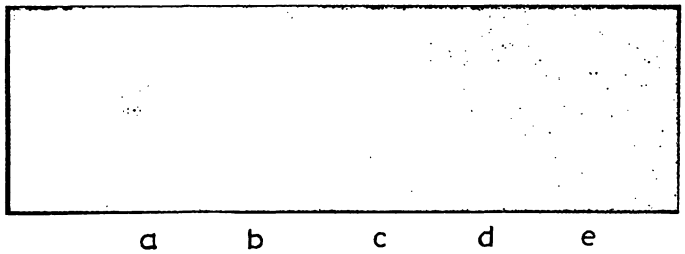

Fig. 5. Photographic detection of a protein blot on nitrocellulose filter.
For detail see Methods.
a, 50 ng; b, 5 ng; c, 500 pg; d, 50 pg; e, 5 pg rabbit immunoglobulin G.

References

1. Gershoni, J. M. (1985) Trends Biochem. Sci., TIBS *10*, 103–106.
2. Gupta, R. C. (1982) Clin. Exp. Immunol. *49*, 543–551.
3. Erlich, H. A., Rodgers, G., Vaillancourt, P., Araujo, F. G. & Remington, J. S. (1983) Infect. Immun. *41*, 683–690.
4. Hall, W. W. & Choppin, P. W. (1981) N. Engl. J. Med. *304*, 1152–1155.
5. Gordon, J., Towbin, H. & Rosenthal, M. (1982) J. Rheumatol. *9*, 247–252.
6. Kühnl, P., Seidl, S. & Holzberger, G. (1986) Vox Sang. *51*, 15–20.
7. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. USA *76*, 4350–4354.
8. Renart, J., Reiser, J. & Stark, G. R. (1979) Proc. Natl. Acad. Sci. USA *76*, 3116–3120.
9. Bowen, B., Steinberg, J., Laemmli, U. K. & Weintraub, H. (1980) Nucleic Acids Res. *8*, 1–20.
10. Towbin, H. & Gordon, J. (1984) J. Immunol. Meth. *72*, 313–340.
11. McMichael, J. C., Greisinger, L. M. & Millman, I. (1981) J. Immunol. Meth. *45*, 79–94.
12. Billings, P. B., Hoch, S. O., White, P. J., Carson, D. A. & Vaugham, J. H. (1983) Proc. Natl. Acad. Sci. USA *80*, 7104–7108.
13. Hawkes, R. (1982) Anal. Biochem. *123*, 143–146.
14. Kyhse-Andersen, J. (1984) J. Biochem. Biophys. Meth. *10*, 203–209.
15. Dao, M. D. (1985) J. Immunol. Meth. *82*, 225–231.
16. O'Connor, C. G. & Ashman, L. K. (1982) J. Immunol. Meth. *54*, 267–271.
17. Jalkanen, M. & Jalkanen, S. (1983) J. Clin. Lab. Immunol. *10*, 225–231.
18. Brada, D. & Roth, J. (1984) Anal. Biochem. *142*, 79–83.
19. Hsu, Y. H. (1984) Anal. Biochem. *142*, 221–225.
20. Turner, B. M. (1983) J. Immunol. Meth. *63*, 1–6.
21. Moeremans, M., Daneels, G. & de Mey, J. (1985) Anal. Biochem. *145*, 315–321.
22. Geiger, R., Stuckstedte, U. & Fritz, H. (1980) Hoppe Seyler's Z. Physiol. Chem. *361*, 1003–1016.
23. Geiger, R., Junk, A. & Jochum, J. (1985) J. Clin. Chem. Clin. Biochem. *23*, 821–828.
24. Miska, W. & Geiger, R. (1987) J. Clin. Chem. Clin. Biochem. *25*, 23–30.
25. Mann, K., Göring, W., Lipp, B., Karl, H. J., Geiger, R. & Fink, E. (1980) J. Clin. Chem. Clin. Biochem. *18*, 395–401.
26. Geiger, R. & Miska, W. (1987) J. Clin. Chem. Clin. Biochem. *25*, 31–38.
27. Lin. W. & Kasamatsu, H. (1983) Anal. Biochem. *128*, 302–311.
28. Bjerrum, O. J. & Schafer-Nielsen (1986) in Electrophoresis 1986 (Dunn, M. J., ed.) VCH Weinheim, Bergstraße.
29. R. Geiger, R. Hauber, unpublished results.

Priv. Doz. Dr. Reinhard Geiger
Abteilung für Klinische Chemie
und Klinische Biochemie
in der Chirurgischen Klinik Innenstadt
der Universität München
Nußbaumstraße 20
D-8000 München 2